Density alteration in non-physiological cells

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Abstract

In present study an important phenomenon of cells was discovered: the change of intracellular density in cell's response to drug and environmental factors. For convenience this phenomenon is named as "density alteration in non-physiological cells" (DANCE). DANCE was determined by discontinuous sucrose gradient centrifugation (DSGC), in which cells were separated into several bands. The number and position of the bands in DSGC varied with the change of cell culture conditions, drugs, and physical process, indicating that cell's response to these factors was associated with alteration of intracellular density. Our results showed that the bands of cells were molecularly different from each other, such as the expression of some mRNAs. For most cells tested, intracellular density usually decreased when the cells were in bad conditions, in presence of dugs, or undergoing pathological changes. However, unlike other tissue cells, brain cells showed increased intracellular density in 24 hrs after the animal death. In addition, DANCE was found to be related to drug resistance, with higher drug-resistance in cells of lower intracellular density. Further study found that DANCE also occurred in microorganisms including bacteria and fungus, suggesting that DANCE might be a sensitive and general response of cells to drugs and environmental change. The mechanisms for DANCE are not clear. Based on our study the following causes were hypothesized: change of metabolism mode, change of cell membrane function, and pathological change. DANCE could be important in medical and biological sciences. Study of DANCE might be helpful to the understanding of drug resistance, development of new drugs, separation of new subtypes from a cell population, forensic analysis, and importantly, discovery of new physiological or pathological properties of cells.

Key words: DANCE, DSGC, intracellular density, drug resistance, cell response

In a previous study we found that RT-PCR results varied in a significantly great range. The poor repeatability of the RT-PCR results could not be explained by the standard deviation of the method. Even under same experimental conditions cells responded quite differently to a same drug. To find out if there were different cells in a cell line with different sensitivities to a drug, we tried to

separate human acute leukemia cell line (K562) by using discontinuous sucrose gradient centrifugation (DSGC). As expected, the cells under normal culture conditions were separated into two bands, which stop at the top of the layer of 50% and the layer of 60% (w/v) sucrose, respectively (Fig 1, A1), and the cells in "bad conditions" (after more than 20 passages) showed two more bands at 30% and 40% sucrose layers (Fig 1, A2). The result indicated that a cell line under same conditions existed in different physiological or pathological statuses with different intracellular densities. From these experiments we found that in a cell population the difference in intracellular density of cells was associated with different drug responses and with different expression levels of some genes.

For convenience, the intracellular density change due to drugs or environmental changes was named as "Density Alteration in Non-physiological Cells", or DANCE. "Non-physiological cells" were the cells under non-physiological or non-naturally living conditions, or those that were undergoing pathological changes. A band in DSGC was labeled as "S" with the number of sucrose concentration at which the cells stop. For example, if a band of cells stop at the top of a layer of 50% (w/v) sucrose it was labeled as "S50", and so on.

To investigate the effects of culture conditions on DANCE, we first determined the influence of culture time (without change of medium) on K562 cells. The cells were continuously cultured for 2, 4 and 6 days, at 37^{o}C, 5% CO_2, 98% humidity, and then separated by DSGC. We found that with increase of incubation time the "heavier" band, "S60", became smaller on day 4 and disappeared on day 6. However, the "lighter" band "S50" became bigger on day 4 and 6, and there was a new band appeared at "S40" on day 6 (Fig 1, B1-3), suggesting that longer time of continuous incubation caused decrease in intracellular density, a phenomenon so called "DANCE up". In addition, seeding concentration of cells was found to have significant effect on DANCE. When the seeding concentrations of K562 cells were 2.5×10^5/ml, 5×10^5/ml and 10^6/ml, cultured for two days, we found that intracellular density decreased ("DANCE up") with increase of seeding concentration of the cells, i.e. only band S60 was found for 2.5×10^5/ml, both bands S50 and S60 were found for 5×10^5/ml, but only band S50 for 10^6/ml (Fig 1, C1-3). It seemed that higher seeding concentrations in culture caused "lighter change" of the cells for bigger living space, or more cells in the culture produced more metabolic wastes that might cause decrease in intracellular density of the cells.

Normally K562 cells could be separated into two bands by DSGC, S50 and S60. We wanted to know if these two bands of cells could change to each other. We isolated the cells from bands S50 and S60 (Fig 1, A1) and separately re-cultured the cells in a new medium. After two days the cells of band S50 generated a new band at S60 (Fig 1, D1) while the cells of band S60 remained at the same level (Fig 1, D2). However, when the cells from bands S50 and S60 (A1) were cultured for 5 days with a change of culture medium at day 2, the cells of band S60 generated a new band at S50 (Fig 1, D4), suggesting that the cells of bands S50 and S60 could change to each other but the cells of S60 remained at the stage for longer time than S50.

To study the cell cycle stages of the cells from different bands, we separately collected the cells from bands S30, S40, S50 and S60 (Fig 1, A2), and determined their DNA contents and apoptosis by flowcytometry. The results showed that percentage of cells at phase S was 53.9%, 64.5%, 55.8% and 50.9%, respectively, while those at phase G_1/G_0, 41.9%, 33.4%, 42.4%, 46.0%, respectively (Fig 1, E1-4, Table 1), i.e. more cells of S40 were found to be at S phase and more

cells of S60 were found at G_1/G_0 phase. Percentage of cells at late apoptosis (UR) was found to be: 16.11%, 12.12%, 11.05%, 6.6%, while those at early apoptosis (LR): 21.92%, 19.82%, 8.31%, 6.47%, and those of live cells (LL): 61.41%, 67.83%, 80.56%, 86.82%, respectively (Fig 1, F1-4, Table 1). The flowcytometric results suggested that more cells undergoing apoptosis with decrease in intracellular density. In other wards, apoptotic cells increased during their "moving up" in the culture. However, most cells of all the four bands were found to be alive. These results raised another question: did these cells with different intracellular densities have different proliferation ability or viability? We determined the cells of the four bands (Fig 1, A2) for their growth curves. Our results showed that cells of S60 and S30 had higher proliferation activity, followed by S50, while those of band S40 showed the lowest activity. However, the results demonstrated that all the four bands had viable cells that could undergo normal process of proliferation.

DANCE phenomenon was found to be significantly associated with cell response to drugs. When K562 cells were incubated with retinoic acid for 48 hr, at 0, 4, 8, 16 μg/ml, "DANCE up" (decrease in intracellular density) was observed with increasing of the drug concentration (Fig 2, A1-4). However, only the cells of band S30 survived at the high concentration (16 μg/ml) of the drug (Fig 2, A4). It is interesting to know that cells of bands S30, -40, -50 and -60 were still alive and kept their proliferation ability in despite of the decrease of intracellular density caused by retinoic acid (Fig 2, F). Because retinoic acid was found to be able to inhibit proliferation of leukemia cells [1,2], when the cells were incubated with the drug, the remained cells might start the process of apoptosis or differentiation, and decrease of intracellular density could be one of the mechanisms associated with the pathological change in cells. Our results showed that when K562 cells were incubated with retinoic acid at 8 μg/ml, cells of band S40 and S50 significantly decreased at G_1/G_0 and S stages, but greatly increased at G_2/M stages (Table2), suggesting that the late stage of cell division could be arrested by the drug, accompanied by decrease of intracellular density. The same change was also observed in LOVO cell line incubated with fluorouracil (5-FU).

When the concentrations of 5-FU were 50, 100 μg/ml ,the band S60 disappeared while the cells of S50 increased, and a small new band appeared at S40 (Fig 2, B1-3), indicating that the cells of S60 were more sensitive to the drug. Because recent studies showed that 5-FU (50 μg/ml) caused increase of stem-like cells by 10 times in a leukemic cell line KG1a [3], this result might suggest that cancer stem cells or stem-like cells mostly stand in the band S50. The band S50 was also found to be more resistant to hydrogen peroxide (H_2O_2) than S60 (Fig 2, D1-4). H_2O_2 had been demonstrated to be able to cause apoptosis of cells [4]. When the concentration of H_2O_2 was 0.25 μg/ml, band S60 disappeared; in contrast, band S50 was still observed at 0.5 μg/ml of H_2O_2. However, no band was observed at S40 or S30, indicating that the action mode of H_2O_2 is different from that of retinoic acid. These results suggested that change of intracellular density could be a general response of cells to drugs or chemical reagents.

In the present study, most chemicals or drugs tested were found to be able to cause decrease in intracellular density, or "DANCE up". However, K562 cells cultured without serum were found to shift down to the heavier band, from S50 to S60 (Fig 2, C1-2), and these cells showed more sensitive to retinoic acid compared to the cells of band S50 and S60 cultured with 10% bovine serum, because the IC_{50} for band S50 and S60 of cells cultured with serum was determined to be 5.0 μg/ml while that for "serum free" cells, 2.5 μg/ml (Fig 2, G). From DNA content analysis we found that most K562 cells cultured without serum were at S and G_2/M stages (Table 2), which

might suggest that "DANCE down" in K562 cells cultured without serum was resulted from the increase of proteins and nucleic acids in the cells due to delay of division. Combined with the results of other drugs or chemicals tested so far, we might suggest that cells with less intracellular density be more resistant to drugs.

"DANCE down" was also observed in K562 cells recovered from cryopreservation (Fig 2, E2), in which 5 bands, S30, S40, S50, S60 and S70, were observed after thawing. Growth curves showed that cells (at least part) of each of the five bands had proliferation activity when they were cultured under normal conditions, and it was found that cells of band S60 and S70 were more active than those of other bands (Fig 2, H), suggesting that the better cryopreserved cells might be those with higher intracellular density during freezing. Because dehydration occurs in cells during freezing, it is reasonable to suggest that "DANCE down" of the cells (S70) could be due to the increased concentrations of matters in cells, especially that of macromolecules and mineral ions. It is interesting to know that K562 cells frozen in the presence of 10% DMSO showed similar bands as the normal K562 cells, i.e. mainly S50 and S60 observed (Fig 2, E3). This might be a reason for DMSO to be used for cell cryopreservation.

To find out if DANCE also occurs in microorganisms, we used bacterium *E. coli* BL21 and fungus *Candida albicans* ATCC10231 as tested models. Compared to K562 cells, *E. coli* BL21 mostly presented in bands S10 and S20, with less bacteria located at S50 and S60 (Fig 3, A2). Furthermore, the bands changed with incubation time. After 24 hrs of incubation, the bacteria mainly appeared at band S20 and small amount at band S60. With longer incubation time, new bands appeared sequentially at S50, S40 and S30 (Fig 3, C1-3). The change of band position might indicate the process of maturation of the new born bacterial cells, that is, bacteria of S10-S20 could be matured cells while those of S60-S30 should be the new born and growing bacterial cells.

To study the response of *E. coli* BL21 to antibiotics, we incubated the bacteria with ampicillin, which inhibits peptidoglycan synthesis in bacterial cell walls. When the concentrations of the drug were 0, 3.125, 6.25 and 12.5 μg/ml and incubation time was 48 hrs, we found that the bacterial cells with higher intracellular densities, i.e. bands S60-S30, disappeared at 6.25 μg/ml while the band S10 remained in the culture (Fig 3, B1-4), indicating that the bacteria of band S10 were more resistant to the drug. This result was similar to that of K562 cells to retinoic acid and LOVO cells to 5-FU (Fig 2, A, B). It is interesting to know that the normally cultured bacteria of bands S10-20, S40, S50 and S60 (Fig 3, B1) showed similar proliferation activities (data not shown). However, those bacteria of S10 cultured with ampicillin at 0, 3.125, 6.25 μg/ml showed different growth curves (Fig 3, D), with higher activity for S10 at 3.125μg/ml, followed by S10 at 6.25 μg/ml, while S10 without ampicillin showed lower activity. We then determined the growth curves of different bands of the bacteria incubated at a same ampicillin concentration. We found that when the bacteria were incubated with the drug at 3.125μg/ml, the bacteria of S10 and S60 had higher proliferation activity, followed by S50, while S40 showed the lowest activity (Fig 3, E).

Unlike bacteria, fungi are eukaryotic microorganisms. *C. albicans* ATCC10231 was found to have different responses to different culture conditions. When the fungus was cultured on a potato dextrose agar at different concentrations of ketoconazole [5], the fungal cells cultured without drug were separated into two bands, S70 and S80, with higher intracellular densities than that of K562 cells (Fig 3, F1, 2). However, when the fungus was cultured in DMEM broth it showed similar bands as those of *E. coli* BL21, with the majority of fungal cells in bands S30-S50 (Fig 3, F3). The

antifugal activity of ketoconazole was detected on potato dextrose agar (Fig 3, G1-6) and the fungal cells were analyzed by DSGC. The results showed that with increasing concentration of ketoconazole in the agar gel, the band S70 increased. When the concentration of the drug was 30μg/L, both bands S70 and S80 disappeared and a new band at S50 was generated (Fig 3, H1-5). This result suggested that the fungal cells responded to the drug by decreasing their intracellular density and only those cells of S50 could survive at the high drug concentration. Since the antifungal activity of the drug is the inhibition of ergosterol biosynthesis, DANCE in the ketoconazole treated fungus might be related to the permeability change of the cell membrane.

The above finding of DANCE phenomena in cultured cell lines and microorganisms encouraged us to test it with animal and human cells. Mouse tissues were homogenized in PBS and the cells suspensions were separated by DSGC. The results showed that mouse white blood cells (WBC) and kidney cells were separated into 3 bands, S40, S50 and S60, brain cells into 2 bands, S30 and S40, and pancreas cells into 2 bands, S50 and S60 (Fig 4, A1-4). It is interesting to know that the bands changed with the sampling time after sacrifice of the animal. Generally, samples collected at time 0 and at 4 hrs after death did not change in DANCE, but significantly changed at time 24 and 48 hrs. The brain cells were unexpectedly found to "DANCE down" with time, i.e. the band S30 disappeared and a major new band appeared at S50 and a minor new band appeared at S60 (F4, B1-4). For lung and heart cells, a new band appeared at S40, while the band S60 reduced at time 48 hrs (Fig 4, C1-4, D1-4). The liver and kidney cells showed 3 bands, 2 major bands at S50 and S60, and a minor band at S40. However, the band S60 reduced significantly at time 48 hrs (Fig 4, E1-4, F1-4). In the experiment with human red blood cells (RBC) which were diluted 100× in PBS and kept at room temperature (25^{o}C), 2 bands, S50 and S60, were observed at time 0, but the band S60 disappeared at the time 24 hrs and 48 hrs (Fig 4, G1-3). Considering osmotic pressure change in the solution might cause change of the water content in RBC and result in change of the band position, we then suspended human RBC in NaCl solutions of 0.45%, 0.9% and 1.8%, at room temperature for 4 hrs. RBC at all of the NaCl concentrations, i.e. in hypo-, iso- and hyper-osmotic solutions, were only observed in bands S50 and S60 (Fig 4, H1-3), suggesting that osmotic change in the solution might have little effect on DANCE. The above results suggested that, except brain cells which "DANCE down" with time, most cells generally "DANCE up" with time after death of the body or after the cells were removed from the body, and it was evident at the time of 24 hrs after the animal death.

Except brain cells, most tested human cells including K562 and LOVO cell lines could be separated by DSGC into bands S50 and S60. We used RT-PCR to determine whether human WBC separated from the two bands were molecularly different from each other. Our results showed that the mRNA level of IL-6 was higher in the cells of S60 than that of S50 (Fig 5, A, B). It has been known that in human blood, monocytes and T-lymphocytes are the main cells that produce and release IL-6, and increased production of IL-6 is related to activation and apoptosis of neutrophils during inflammations [6, 7]. Therefore, our results might indicate that the activated monocytes and T-lymphocytes should be mainly located in S60. However, the mRNA level of CD36, a member of the scavenger receptor family, was found to be higher in the cells of S50 than that of S60 (Fig 5, A, B), and Western blot also showed that CD36 protein was much higher expressed in S50 than in S60 (Fig 5, C). Since CD36 has been found to be highly expressed by peripheral blood mononuclear cells in some diseases or inflammations [8], we might suggest that the activated CD36-producing mononuclear cells were mainly located in S50, and the cells producing CD36

might not be the same cells as that producing IL-6, although both IL-6 and CD36 proteins are involved in inflammatory responses in blood. These results indicated that cells at different functional stages might exhibit different intracellular densities.

The mechanisms for DANCE phenomenon are not clear. Based on our present experimental results, we hypothesize the following causes for the alteration of intracellular density:

(1) Change of metabolism mode: when biosynthesis of macromolecules increases, especially proteins and nucleic acids, intracellular density will increase due to accumulation of the condensed "particles" in cells, and in addition, many biosynthesis of macromolecules are accompanied by deposition of metal ions in cells, such as K, Mg, Ca, Zn, etc, which should contribute a part to the intracellular density. This might explain for "DANCE down" of K562 cells when the seeding concentration was low so that the cells were grown in rich of nutrients (Fig 1, C1), and when the culture medium contained no serum so that most cells stood at S and M phases (Fig 2, C2, Table 2). On the other hand, increase in degradation of macromolecules will cause decrease of intracellular density. Many drugs may affect protein biosynthesis and as a result, protein degradation relatively increases in cells, leading to reduction of intracellular particles such as ribosomes and enzyme complexes. This might explain for "DANCE up" in K562 cells when they were treated with retinoic acid (Fig 2, A1-4), and in LOVO cells when they were incubated with 5-FU (Fig 2, B1-3). Cells seeded at high concentrations in culture medium might suffer from a relative "malnutrition", resulting in shift from anabolism to catabolism in cells. This might explain for "DANCE up" in K562 cells seeded at high concentrations (Fig 1, C3). However, it should not be excluded that "DANCE up" in K562 cells seeded at high concentrations was due to higher levels of metabolic wastes or decrease of pH in the medium. On the other hand, increase in biosynthesis of lipids may cause decease in intracellular density, which might explain "DANCE up" of liver cells from a fatty-liver animal model (data not shown). In contrast, increase in degradation of lipids might cause relative increase of intracellular density, which might explain for "DANCE down" of brain cells in 24 hrs after the animal death (Fig 4, B3).

(2) Change in membrane function. One of the most important functions of the cell membrane is to control the movement of materials into and out of the cell and therefore, affect the intracellular density. Efflux and influx of water out of or into cells due to hyper or hypotonic osmosis might play a small effect on DANCE, since a normally functional membrane would not allow too much water to move out of the cells at room temperature, while too much water influx may kill the cells immediately, and this might explain for that no evident DANCE was observed in RBC in hypo- or hyper-osmotic solutions (Fig 4, H1-3). However, shrinking of cells due to efflux of water during freezing could cause significant increase of intracellular density, or "DANCE down", such as the band S70 seen in frozen K562 cells (Fig 2, E2). Degradation or inhibition of biosynthesis in cell membrane might cause dysfunction of the membrane and thus DANCE. This could explain for the deceased intracellular density in *E. coli* by ampicillin and in *C. albicans* by ketoconazole. On the other hand, decrease in energy generation (or lower ATP level) might result in dysfunction of the cell membrane, cause leaking of intracellular mineral ions especially K and Ca, and therefore, "DANCE up". Degradation or malfunction of mitochondria might cause "DANCE up" through the effect on the membrane function. This might occur when the cells were undergoing apoptosis, such as the bands S30 and S40 seen in K562 cells in bad conditions (Fig 1, A1, Table 1).

(3) Pathological change of the cells. Pathological change in cells may be a complex process depending on the pathogenic factors. However, most (if not all) pathological changes may more or less result in change of metabolism in cells, including alteration of biosynthesis and degradation of macromolecules, and thus energy production might be affected, resulting in dysfunction of the cell membrane. Therefore, DANCE caused by pathological changes might be involved in both changes of metabolism and of cell membrane function, and in each situation one of these changes might be the main cause of DANCE. Pathological changes should occur in all tissue cells 24 hrs after death of the animal. "DANCE down" was observed in brain cells 24 hrs after sacrifice of the mouse (Fig 4, B1-4), suggesting the major metabolic change was degradation of materials especially lipids, which are the most rich components in neural cells. Reduction of lipids in neural cells could be the main cause for increase of density in the brain cells. However, for non-neural cells degradation of macromolecules should cause decrease in intracellular density and thus, "DANCE up", such as cells of lung, heart, liver and kidney in 24 hrs after death of the mouse (Fig 4, C, D, E, F). Furthermore, increase of macromolecule degradation should be associated with decrease of biosynthesis of the same molecules, and membrane dysfunction might occur because of less of ATP due to hypoxia in the tissues. However, "DANCE up" in RBC 24 hrs after collection might be mainly resulted from decrease of membrane function, resulting in leak of intracellular ions and influx of water, since RBC membrane is much more fragile than other cells. This might explain for "DANCE up" in human RBC kept at room temperature (25^{o}C) for 24 hrs (Fig 4, G1-3).

DANCE could be an important phenomenon in medical and biological sciences. First, drug resistance of cancer cells and microorganisms might be related to DANCE. Our results showed that cells of band S60 in both K562 and LOVO cell lines were more sensitive to drugs than that of S50 (Fig 2, A, B), and the most resistant K562 cells against retinoic acid were found to be those of S30 (Fig 2, A4). Furthermore, the similar phenomena were also found in bacteria and fungus, of which only those with decreased intracellular density could survive at high drug concentrations (Fig 3, B1-3, H1-5). However, it is not clear whether "DANCE-up" increased the viability of cells in presence of drugs, or the drugs caused "DANCE-up", or both. Therefore, study of DANCE phenomenon might promote the understanding of the mechanisms for drug resistance, which should benefit the development of new drugs. Second, DANCE phenomenon could be related to cell healthy status, growth, differentiation, aging, and apoptosis. Our results showed that when K562 cells were normally cultured, two bands (S50 and S60) were observed by DSGC, which were mostly "healthy" cells. However, after more than 20 generations and when the cells were cultured at higher seeding concentrations, lots of K562 cells were observed at S30 and S40 (Fig 1, A2), which might be seen as less healthy cells, since flowcytometric analysis showed that a significant amount of S30 and S40 cells were apoptotic or dead (Table 1). When cells of S50 and S60 were separately re-cultured, S50 generated S60 in 48 hrs, but S60 needed more time to generate S50 (Fig 1, D1-4), suggesting that band S50 should be mostly mature (or late G1) cells which were ready for the next cycle, while band S60 should be mostly newly divided (or early G1) cells. The similar phenomenon was also observed in *E. coli* BL21, in which S60 was generated first and should be the newly divided cells, while S50, S40 and S30 be the growing bacterial cells and the cells of S10-20 be the mature ones (Fig 3, C1-3). In addition, cells from different bands should be molecularly different from each other, such as different levels of some mRNAs (e.g. IL-6 and CD36) seen in S50 and S60 in human WBC (Fig 5, A, B, C). This might help scientists

to separate the cells with different physiological properties or drug resistance from a cell population or a cell line for the research of interest. Third, DANCE could be an important parameter for forensic analysis, since in most tissues change of intracellular density was found to be related to the time from death of the body, and DANCE could also reflex the change of environment or presence of drugs or toxins. It is interesting to know that the brain tissue cells sampled in 4 hrs after death of the mouse did not show DANCE (Fig 4, B2). Did this result implicate that life could be saved even in 4 or more hrs after heartbeat stop? Finally, in a cell population, DANCE should vary in a certain range, in which cells stay with a series of different intracellular densities. This might means that by using different medium gradients more bands of cells could be separated and more specific properties of cells could be discovered.

Summary of Methods

Cell culture

K562 and LOVO cell lines were cultured in RPMI1640 and DMEM respectively, with 10% bovine serum, 5% CO_2, 98% humidity, at 37^{o}C. The culture medium was changed every 3 days except otherwise indicated. LOVO cells were digested with trypsin for 3 min before collection. The cells were centrifuged at 500g for 5 min to remove the culture medium and re-suspended in PBS for DSGC.

Culture of bacteria and fungus

E. coli BL21 was cultured in LB broth at 37^{o}C with shaking, with or without ampicillin. The bacteria were collected by centrifugation at 1600g for 10 min and re-suspended in PBS before DSGC. After DSGC, the bands of bacteria were separately collected and washed 2 times in PBS for further test. Growth curves were measured by determination of the OD at 600nm of the culture at different time points. For culture of the fungus ATCC10231, potato dextrose agar with different concentrations of ketoconazole was prepared in 12 well-plates [5]. About 10 μl of the fungus suspension was inoculated on each well and incubated at 30^{o}C for 3 days. The fungal cells were collected by washing the colonies with distilled water and the fungal cell suspensions were directly used for DSGC.

Animal tissue cells

Three mice were sacrificed by cervical dislocation. At the time points 0, 24 and 48 hrs, each of the mouse was dissected and the organs were collected and washed with PBS. About 200 mg of each organ tissue was homogenized in 1 ml PBS at room temperature, and the homogenates were immediately separated by DSGC. For time course analysis of human RBC, the whole blood sample with EDTA as anticoagulant was stored at room temperature for 0, 24, 48 hrs. At the end of each time point an aliquot of the blood was diluted 100 times in PBS, of which 0.2 ml was used for DSGC. For the effect of osmotic pressure on DANCE, the human whole blood with anticoagulant was centrifuged to remove plasma and washed twice in saline. The RBC cells were re-suspended in NaCl solutions of 0.45%, 0.9% and 1.8% (w/v), at a final RBC concentration of 3×10^{7}/ml. The RBC suspensions were kept at room temperature (25^{o}C) for 4 hrs before DSGC. WBC of human or mouse were separated by using lymphocytes separation medium and re-suspended in PBS before DSGC.

Discontinuous sucrose gradient centrifugation

Sucrose solutions of different concentrations (w/v) were freshly made in distilled water before use. About 550 μl of each solution from high to low concentrations was gently loaded into a glass tube

with a radius of 5 mm. For K562, LOVO cells and animal cells, the sucrose concentration gradients were usually made as 4 layers (except otherwise indicated): 60%, 50%, 40% and 30%. For *E. coli* BL21, 6 layers from 60% to 10%, and for *C. albicans* ATCC10231, 6 layers from 80% to 30% (or from 90% to 40%). About 200 μl of cells suspension was loaded on the top layer of the gradient and centrifuged at 1600 g for 5 min. When the bands were used for further tests, the cells of each band were collected separately and washed twice in PBS.

Flowcytometric analysis

Cells separated from DSGC were washed twice in PBS to remove sucrose. For DNA content measurement by flowcytometry, the cell pellets were fixed in 75% ethanol overnight before analysis. For apoptosis determination the cells were re-suspended in saline. The samples were determined by flowcytometry according to the instructions of the machine.

Growth curve determination

Cells separated from DSGC were washed twice in PBS and then seeded in RPMI1640 with 10% bovine serum into a 96-well plate, with 5000 cells/well in triplicates. The plate was cultured at 37^{o}C, 5% CO_2 and 98% humidity. Count the cells of each well in every 24 hrs. The cell numbers were divided by that of day 1 in order to normalize the growth rates. Plot the growth curve with normalized growth rates versus the incubation days.

MTT assay

K562 cells were cultured with and without 10% bovine serum for 48 hrs and separated by DSGC as described above. Two bands, S50 and S60 were obtained from the culture with 10% bovine serum, but only band S60 was obtained from the culture without bovine serum. Cells of the three bands were collected and washed twice in PBS. The cells were transferred into a 96-well plate with 5000 cells/well. The plate was incubated at 37^{o}C, 5% CO_2 and 98% humidity for 24hrs, and then added retinoic acid to final concentrations of 0, 0.75, 1.5, 3.0, 6.0, 12.0, 24.0 μg/ml, in triplicates for each concentration. After incubation for 24 hrs, 20 μl/well of MTT solution (5 mg/ml) was added and the plate was incubated at 37^{o}C for another 4 hrs. At the end of incubation the supernatant was removed and 150 μl/well of DMSO was added. Shake the plate for 10 min, read the OD_{570} of each well, and plot the curves with OD_{570} versus drug concentration for determination of IC_{50}.

RT-PCR

Human blood samples with EDTA as anticoagulant were collected from out-patients in the Overseas Chinese Hospital, Guangzhou, China. WBC was separated by using lymphocytes separation medium. The separated WBC was washed twice and re-suspended in PBS before DSGC. Cells collected from S50 and S60 were extracted for total RNAs with Trizol reagent and the cDNA of each sample were produced by PCR according to the instruction. mRNA levels of β-actin, CD_{36} and IL-6 were determined by PCR. The primers were synthesized by Invitrogen Co. Shanghai, China. The primer's sequences and the PCR product sizes are: for β-actin, forward: TGG GTC AGA AGG ATT CCT ATG T ,reverse :CAG CCT GGA TAG CAA CGT ACA, 276 bp; for CD36, forward: GAG AAC TGT TAT GGG GCT AT, reverse: TTC AAC TGG AGA GGC AAA GG, 387 bp; for IL-6, forward: AAA GAG GCA CTG GCA GAA AA, reverse: AAC AAC AAT CTG AGG TGC CC, 419 bp; The reaction mixture consisted of 10× PCR buffer 2.5μl, dNTP 1 μl, primer 0.75 μl/each, Taq polymerase 0.5 μl, cDNA 2 μl, DDH_2O_2 17.5 μl. PCR was run at 94^{o}C for 5 min, followed by 35 cycles with each as: 94^{o}C for 30s, 55^{o}C for 30s, 72^{o}C for 60s. The

reaction was ended by an extension at 72°C for 10 min. 5 μl of each PCR product was analyzed by 2% agar gel electrophoresis.

Western blot

Human WBC was separated from whole blood samples by using lymphocytes separation medium, and DSGC was carried out to obtain the cells of bands S50 and S60. Proteins were extracted with cell lysis buffer. 6 μl of each of the cell lysis mixtures was separated by SDS-PAGE. Western blot was carried out with the primary antibodies against human CD36 and β-actin respectively.

References

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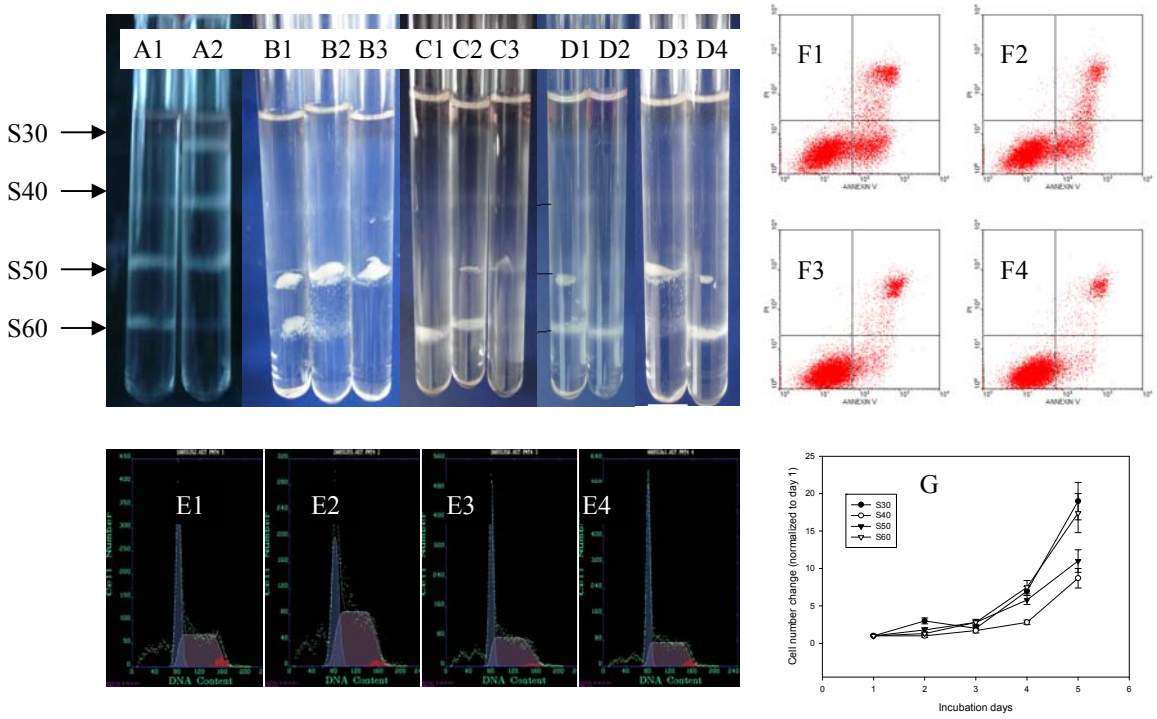
Fig 1 DANCE phenomena in K562 cells responding to changes of culture conditions



A1-2: Normally cultured K562 cells (A1) and K562 cells in bad conditions with visible dead cells (A2).

B1-3: K562 cells were continuously cultured for 2, 4, 6 days respectively.

C1-3: K562 cells were cultured for two days with seeding concentrations of 2.5×10^5/ml, 5×10^5/ml and 10^6/ml, respectively;

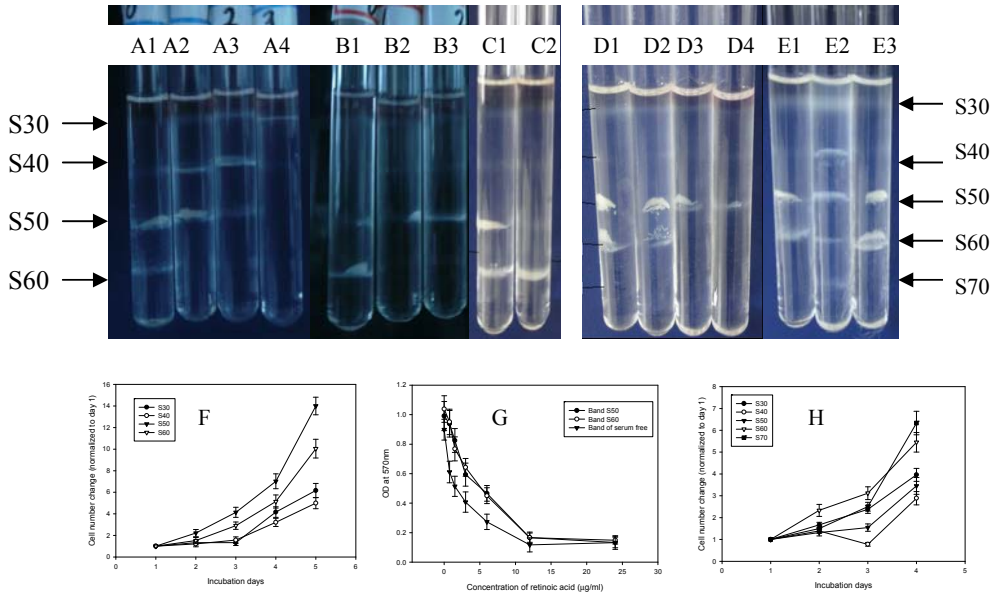
D1-2: K562 cells of bands S50 and S60 (A1) were separately re-cultured for 2 days; D3-4: K562 cells of bands S50 and S60 (A1) were separately re-cultured for 5 days with one change of culture medium on day 2.

E1-4: Flowcytometric analysis of DNA contents for K562 cells of band S30, S40, S50 and S60 (A2), respectively. The parameters were listed in table 1.

F1-4: Flowcytometric analysis of apoptosis for K562 cells of band S30, S40, S50 and S60 (A2), respectively. The parameters were listed in table 1.

G: Growth curves (n=3) of K562 cells from the four bands ofA2.

Fig 2. DANCE phenomena in K562 cells in response to drugs and physicochemical factors



A1-4: K562 cells incubated with retinoic acid for 48hr, at 0, 4, 8, 16 μg/ml, respectively.

B1-3: LOVO cells incubated with 5-FU for 48hr, at 0，50，100 μg/ml，respectively.

C1-2: K562 cells incubated with (C1) and without (C2) bovine serum for 2 days.

D1-4: K562 cells incubated with H_2O_2 for 48hr, at 0, 0.12, 0.25, 0.5 μg/ml, respectively.

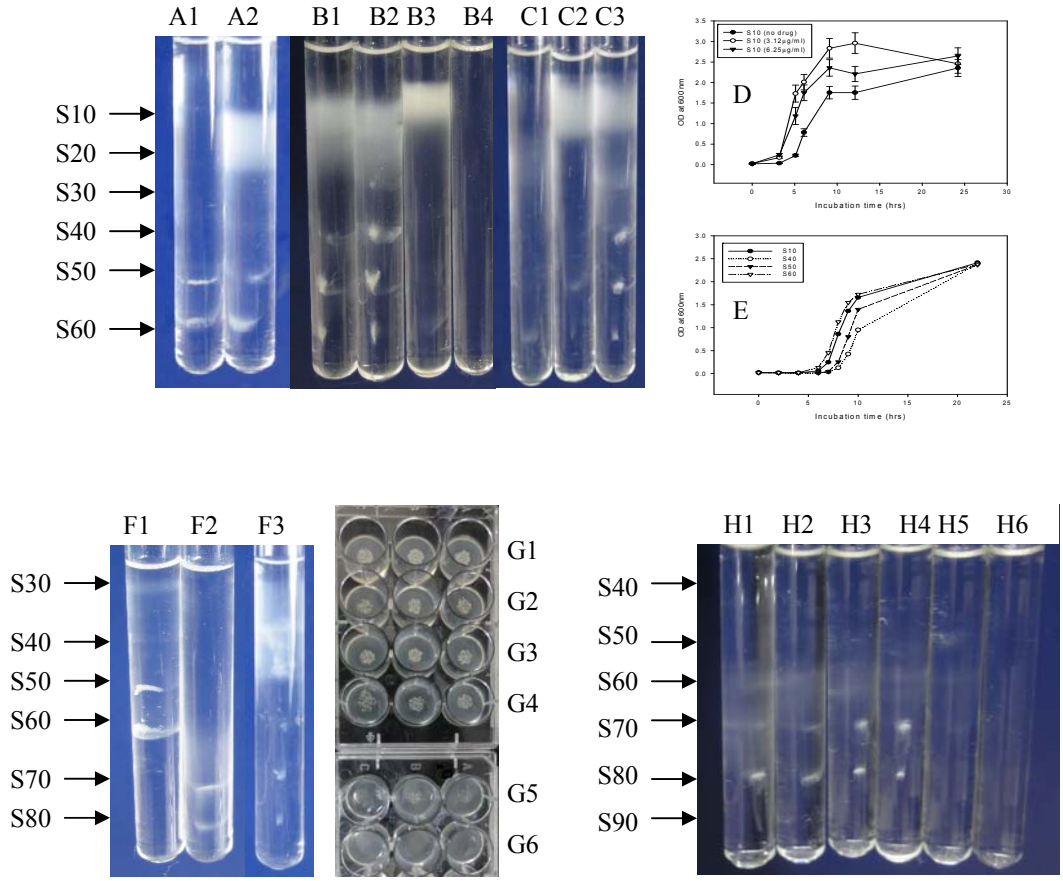
E1-3: Normally cultured K562 cells (E1) and cells thawed from frozen K562 cells without (E2) and with (E3) 10% DMSO (stored at -70^{o}C for 7 days).

F: Growth curves (n=3) of K562 cells from band S30, S40, S50 and S60 (A3), respectively, which were incubated with retinoic acid at 8 μg/ml for 2 days.

G: MTT determination (n=3) for IC_{50} of retinoic acid against K562 cells of band S50, S60 (C1) and serum free cells (C2). The IC_{50} for band S50 and S60 was determined to be 5.0 μg/ml and that for serum free cells, 2.5 μg/ml.

H: Growth curves (n=3) of K562 cells of bands S30-S70 (E2), respectively, which were recovered from frozen K562 cells without DMSO.

Fig 3 DANCE phenomena in microorganisms in response to drugs.



A1-2: K562 cells (A1) and *E. coli* BL21 (A2) separated by DSGC.

B1-4: *E. coli* BL21 incubated with ampicillin for 48hrs, at 0, 3.125, 6.25, 12.5 μg/ml, respectively.

C1-3: *E. coli* BL21 normally cultured for 24, 48, 72 hrs, respectively.

D: Growth curves（n=3）of *E. coli* BL21 collected from S10 with ampicillin at 0, 3.125, 6.25 μg/ml (B1-3), respectively.

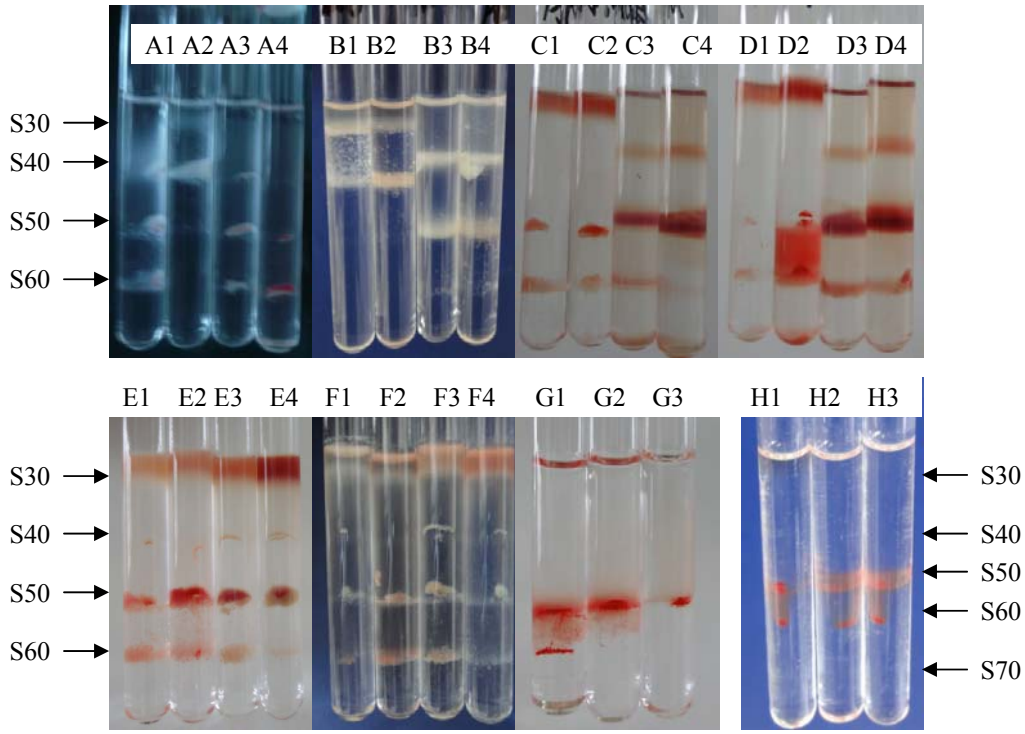
E: Growth curves （n=3）of *E. coli* BL21 from S10, S40, S50, S60 with ampicillin at 3.125 μg/ml (B2), respectively.

F1-3: Normally cultured K562 cells (F1), *C. albicans* ATCC10231 cultured on PDA (F2) and in DMEM (F3), respectively

G1-6: *C. albicans* ATCC10231 incubated on PDA with ketoconazole at 0, 5, 10, 20, 30, 40 μg/L, respectively.

H1-6: DSGC separation for *C. albicans* ATCC10231 collected from PDA cultures (G1-6) with ketoconazole at 0, 5, 10, 20, 30, 40 μg/L, respectively.

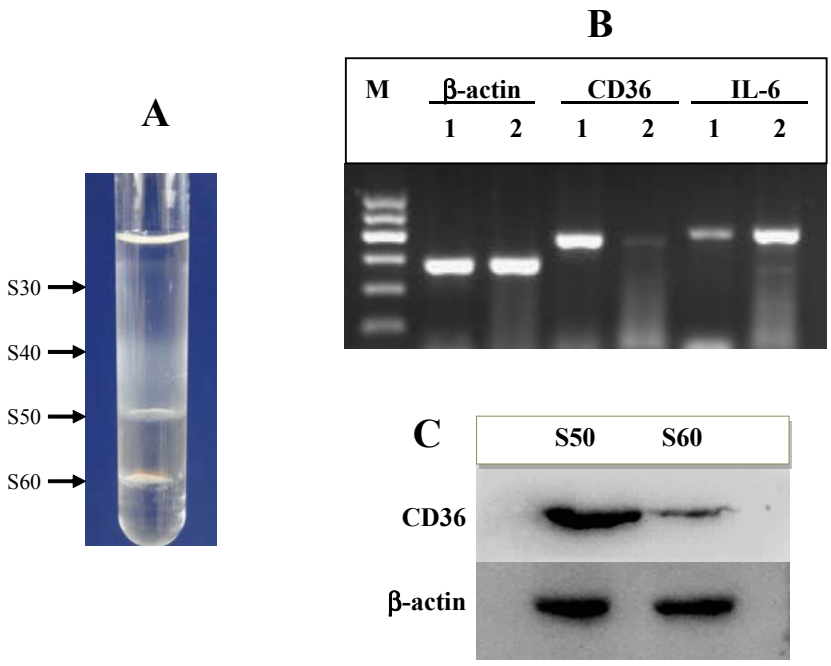
Fig 4 DANCE phenomena in mammalian cells in response to sampling time.



(note: red color at S30 was hemolisate)

A1-4: Mouse tissue cells from WBC, brain, kidney, and pancreas, respectively.
B1-4: Mouse brain cells sampled at 0, 4, 24 and 48hrs after sacrifice, respectively.
C1-4: Mouse lung cells sampled at 0, 4, 24 and 48hrs after sacrifice, respectively.
D1-4: Mouse heart cells sampled at 0, 4, 24 and 48hrs after sacrifice, respectively.
E1-4: Mouse liver cells sampled at 0, 4, 24 and 48hrs after sacrifice, respectively.
F1-4: Mouse kidney cells sampled at 0, 4, 24 and 48hrs after sacrifice, respectively.
G1-3:Human RBC with anticoagulant kept at room temperature (25^{o}C) for 0, 24 and 48 hrs after collection, respectively.
H1-3: Human RBC suspended in NaCl solutions of 0.45% 0.9%, 1.8% respectively, at room temperature (25^{o}C) for 4 hrs.

Fig 5 Separation of human WBC and RT-PCR analysis for the bands S50 and S60



A: Separation of human WBC by DSGC. Bands S50 and S60 were collected for RT-PCR analysis

B: RT-PCR analysis for mRNA levels of β-actin, CD36 and IL-6 in human WBC (A). M: DNA lader (100, 200, 300, 400, 500, 600 bp); 1: cells of S50; 2: cells of S60.

C: Western blot analysis for β-actin and CD36 in WBC of S50 and S60.

Table 1 Flowcytometric analysis for K562 cells* separated by DSGC (referred to Fig 1, A2)

Band	G_1/G_0	S	G_2/M	Live	Apoptotic	Dead
S30	41.9	53.9	4.2	61.4	21.9	16.1
S40	33.4	64.5	2.1	67.8	19.8	12.1
S50	42.4	55.8	1.7	80.6	8.3	11.0
S60	46.0	50.9	3.1	86.8	6.5	6.6

The cells were in bad conditions with visible dead cells.

Table 2 Flowcytometric analysis of cell cycle for K562 cells separated by DSGC (n=3)

Band	G_1/G_0	S	G_2/M
S50	56.4 ±3.9	38.1 ±2.1	5.4 ±5.1
S60	54.2± 3.9	41.9 ±1.1	3.9 ±2.9
S60（SF）	23.2 ±4.2 *	57.6 ± 3.6*	19.3 ±6.7*
S40（VA）	48.6 ± 3.8**	23.2 ±3.1**	28.2 ±6.2**
S50（VA）	45.0 ± 4.2**	27.4 ± 2.7**	27.7 ±5.3**

*P<0.05 when compared to S60; ** P<0.05 when compared to S50. SF: serum free; VA: retinoic acid = 8μg/ml